Neuregulin-1 and ErbB4 Immunoreactivity Is Associated with Neuritic Plaques in Alzheimer Disease Brain and in a Transgenic Model of Alzheimer Disease

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Abstract. Neuregulin-1 (NRG-1) regulates developmental neuronal survival and synaptogenesis, astrocytic differentiation, and microglial activation. Given these NRG-1 actions, we hypothesized that the synaptic loss, gliosis, inflammation, and neuronal death occurring in Alzheimer disease (AD) is associated with altered expression of NRG-1 and its receptors (the erbB2, erbB3, and erbB4 membrane tyrosine kinases). We examined the expression and distribution of NRG-1 and the erbB kinases in the hippocampus of AD patients and cognitively normal controls and in transgenic mice that coexpress AD-associated mutations of the β amyloid precursor protein (APP\textsubscript{K670N,M671L}) and presenilin-1 (PS1\textsubscript{M146L}). In the hippocampi of both control humans and wild type mice, NRG-1 and the 3 erbB receptors are expressed in distinct cellular compartments of hippocampal neurons. All molecules are associated with neuronal cell bodies, but only NRG-1, erbB2, and erbB4 are present in synapse-rich regions. In AD and in the doubly transgenic mouse, erbB4 is expressed by reactive astrocytes and microglia surrounding neuritic plaques. In AD brains, microglia and, to a lesser extent, dystrophic neurites, also upregulate NRG-1 in neuritic plaques, suggesting that autocrine and/or paracrine interactions regulate NRG-1 action within these lesions. NRG-1 and erbB4, as well as erbB2, are similarly associated with neuritic plaques in the doubly transgenic mice. Thus, in AD the hippocampal distribution of NRG-1 and erbB4 is altered. The similarities between the alterations in the expression of NRG-1 and its receptors in human AD and in APP\textsubscript{K670N,M671L}/PS1\textsubscript{M146L} mutant mice suggests that this animal model may be very informative in deciphering the potential role of these molecules in AD.

Key Words: Acetylcholine receptor inducing activity; Astrocytes; Dystrophic neurites; Glial growth factor; Microglia; Neuregulin-1.

INTRODUCTION

Alzheimer disease (AD), the most common cause of dementia, is characterized by widespread abnormalities of the neuronal cytoskeleton (neurofilibrillary tangles) and extracellular accumulations of abnormally processed, insoluble amyloid β-peptide (A\textsubscript{β}42/43) associated with dystrophic neurites, reactive astrocytes, and reactive microglia (neuritic plaques). Although the mechanisms underlying AD are incompletely understood, mutations of the amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2) genes are known to contribute to the pathogenesis of AD in a subset of patients with early-onset, familial autosomal dominant (FAD) forms of the disease (1). These mutations likely lead to overproduction of A\textsubscript{β}42/43 and deposition of this peptide in the neural parenchyma (the “amyloid cascade” hypothesis). Consistent with this hypothesis, plaques histologically similar to those in human AD are present in the cerebral cortex of transgenic mice that overexpress mutant APP (2–6). Transgenic mice that express an FAD-linked PS1 mutation have only modest elevations in A\textsubscript{β}42/43 levels and very few plaques (7, 8); however, transgenic mice that coexpress a mutated form of PS1 (PS1\textsubscript{M146L} or PS1\textsubscript{A246E}) and a mutant APP gene (APP\textsubscript{K670N,M671L}, isolated from a Swedish FAD kindred) demonstrate rapid deposition of amyloid and develop AD-like plaques several months before singly transgenic APP animals (9, 10). Doubly transgenic (PS1\textsubscript{M146L}/APP\textsubscript{K670N,M671L}) mice also show other abnormalities, including a reduction in the density and size of cholinergic synapses in cerebral cortex and hippocampus (11, 12).

The mechanisms by which amyloid deposition induces synaptic loss, gliosis, inflammatory responses, neuronal death, and other pathologic changes characteristic of AD remain poorly understood. These abnormalities may occur, in part, because amyloid deposition and subsequent plaque formation interferes with the actions of growth and differentiation molecules such as members of the neuregulin-1 (NRG-1) family. The NRG-1 proteins are structurally related polypeptides that are translated from alternatively spliced mRNAs transcribed from the neuregulin-1 locus (13). These factors have multiple actions during development, including a well-established role in the formation and maintenance of developing neuromuscular junctions, where they regulate the expression of synapse-associated molecules such as sodium channels...
and nicotinic acetylcholine receptor subunits (14). NRG-
1 proteins are believed to play a similar role during syn-
apto genesis in the developing brain (15–17) and in activity-dependent neuronal plasticity (18). Consistent with
this hypothesis, NRG-1 increases neuronal expression of
neurotransmitter receptors including NMDA (17), GA-
BA_1 (16), and nicotinic acetylcholine receptor subunits
(15). NRG-1 also promotes survival (19, 20) and neurite
outgrowth (19) in some neuronal populations. NRG-1 ac-
tions are not limited to neurons; these factors support the
differentiation and survival of astrocytes (21) and may
regulate the activity of reactive microglia (22). Thus,
NRG-1 is an important regulator of several processes that
are affected in AD, including synapse formation, activation
of inflammatory responses (23, 24), gliosis, and neu-
ronal death (1). Further, one of the NRG-1 receptor sub-
units, erbB4, undergoes intramembraneous presenilin-
dependent γ-secretase cleavage (25, 26), suggesting that al-
tered activity of the NRG-1/erbB signaling pathway plays
a role in the pathogenesis of AD.

While most of the NRG-1 effects described above have
been characterized in the developing nervous system, rel-
atively little is known about the expression and functions
of the NRG-1 proteins and their receptors (the erbB2,
erB3, and erbB4 membrane tyrosine kinases) in the
adult human nervous system. Accordingly, it is important to
ascertain whether NRG-1 and their erbB receptors are
expressed in aging human brain and whether the expres-
sion of these molecules is altered in human AD. As an
initial investigation of these questions, we have examined
the expression and distribution of NRG-1 and the erbB
kinases in hippocampus from cognitively normal aging
humans, AD patients, and doubly transgenic mice co-
expressing a mutant presenilin-1 (PS1\text{M146L}) gene and the
mutant APP gene found in Swedish FAD patients (\text{APP}_{K670N,M671L}).

**MATERIALS AND METHODS**

**Autopsy Material**

Protocols for experiments using human tissue were reviewed
and approved by the University of Alabama at Birmingham’s
Institutional Review Board. Hippocampal tissue was obtained
from the Brain Resource Program/Alzheimer’s Disease Re-
search Center Neuropathology Core Laboratory of the Univer-
sity of Alabama at Birmingham. Eleven AD cases (mean age
81.1 ± 6.2 yr [range 72–94 yr]; postmortem interval 7.2 ± 5.5
h [range 3.5–17 h]; mean ± standard error of the mean) and 5
cognitively normal non-Alzheimer disease controls (mean age
74.4 ± 5.5 yr [range 70–80 yr]; postmortem interval 10.7 ±
4.6 h [range 6–15.5 h]) were selected for analysis based on
consensus conference diagnosis and the availability of both fro-
zen and fixed, paraffin-embedded tissue. Hematoxylin- and
eosin-stained sections, modified Bielschowsky preparations, and
ubiquitin immunostains were independently evaluated to con-
firm the diagnosis in each case. Plaque densities were assessed
following the CERAD criteria (27). Cytoskeletal (neurofibril-
lary tangle) pathology was staged using the Braak and Braak
methodology (28).

**Animals and Tissue Preparation**

The Institutional Animal Care and Use Committees of the
University of South Florida College of Medicine and the Uni-
versity of Alabama at Birmingham approved protocols for ex-
periments on transgenic mice and their nontransgenic litter-
mates. Mice were maintained in accordance with the guidelines
of the National Institutes of Health Guide for the Care and Use
of Laboratory Animals.

Transgenic mice coexpressing mutant presenilin-1 (PS1\text{M146L})
and amyloid precursor protein (\text{APP}_{K670N,M671L}) transgenes were
propagated in the animal facilities of the University of South
Florida College of Medicine. Doubly transgenic animals were
identified by PCR analysis of genomic DNA.

Prior to tissue collection, mice were anesthetized with ether
inhalation. They were then given a lethal dose of sodium pen-
tobarbital (100 mg/kg), following which brains were removed
and the 2 hemispheres separated. One hemisphere was immers-
ed in 4% paraformaldehyde in PBS overnight at 4°C;
the other hemisphere was rapidly frozen and stored at ~80°C
until utilized for protein preparation. Fixed hemispheres were
rinsed in PBS, dehydrated through graded alcohols and xylenes,
and paraffin embedded. Eight-μm-thick sections were cut in the
coronal plane, and every tenth section mounted on SuperFrost
Plus slides (Fisher Scientific, Atlanta, GA).

**Antisera and Immunohistochemical Reagents**

Rabbit polyclonal antibodies and corresponding peptides spe-
cific for the NRG-1 receptor subunits erbB2/c-neu (sc-284) and
erbB4 (sc-283) were obtained from Santa Cruz Biotechnology
(Santa Cruz, CA). A rabbit polyclonal antibody specific for
erbB3 (E-38530) was purchased from Transduction Laborato-
ries (Lexington, KY). A rabbit polyclonal antibody specific for
NRG-1 transmembrane isoforms with an “a” carboxy terminus
(sc-348) was obtained from Santa Cruz Biotechnology (Santa
Cruz, CA). A mouse anti-MAP2 antibody (SM52) was pur-
chased from Sternberger Monoclonals (Gaithersburg, MD).
Anti-human CD45 (mouse monoclonal 2B11) was from Ab-
cam. An anti-glial fibrillary acidic protein (GFAP; mouse
monoclonal G-A-5) antibody was obtained from Sigma (St.
Louis, MO). The pre-A4 mouse monoclonal 22C11 was pur-
chased from Chemicon (Temecula, CA). Nonimmune rabbit
and mouse IgGs were obtained from Santa Cruz Biotechnology
and Jackson Immunoresearch Laboratories (West Grove, PA),
respectively. Horseradish peroxidase (HRP)-conjugated donkey
anti-rabbit secondary antibody was from Santa Cruz Biotech-
nology. Tyramide signal amplification reagents, including strep-
tavidin-horseradish peroxidase, streptavidin-fluorescein, bioti-
nyl tyramide, amplification diluent, and blocking reagent were
purchased from Perkin-Elmer Life Science Products (Rena-
sissance TSA-Indirect kit, Boston, MA). DAB peroxidase sub-
strate (SK-4100) was obtained from Vector Laboratories (Burl-
ingame, CA).

**Histochemical and Immunohistochemical Staining**

Modified Bielschowsky stains were performed on paraffin
sections to precisely localize neuritic plaques and neurofibril-
lary tangles. Following staining, sections were dehydrated

through graded alcohols and xylenes and mounted with Permount for examination.

Single label immunohistochemistry for NRG-1 and erbB receptor subunits was performed on paraffin sections using a highly sensitive methodology in which immunoreactivity in paraffin sections is enhanced by antigen rescue coupled with tyramide signal amplification (29). We have previously demonstrated that the distribution of erbB immunoreactivity detected using this methodology coincides with the expression of erbB mRNA detected by in situ hybridization (29). NRG-1-like immunoreactivity was detected using an antibody specific for an epitope at the carboxy terminus of the major group of NRG-1 transmembrane precursors expressed in brain (splice variants containing an "a" carboxy terminus). Immunostaining for erbB2 and erbB4 was performed using antibodies specific for epitopes in the carboxy terminal (autophosphorylation) domains of these tyrosine kinases (residues 1169–1182 and 1291–1308, respectively); the erbB3-specific antibody used in this study recognizes extracellular domain II. These NRG-1 and erbB antibodies have been used in numerous immunohistochemical studies (29) and found to consistently produce specific staining. Primary antibodies were used at the following dilutions: NRG-1, 1:200; erbB2, 1:100; erbB3, 1:500, and erbB4 1:200. To confirm staining specificity, parallel experiments were performed in which the primary antibody was replaced with nonimmune IgG. For blocking studies, antisera were preincubated with 10 ng/ml of either the immunizing peptide or a nonrelated peptide and staining performed as described above. In all instances, staining was specifically blocked when primary antibody was preincubated with the immunizing peptide, but not when it was preincubated with an unrelated peptide.

For double label immunohistochemical experiments, initial specimen processing and staining was performed as described above. NRG-1 and erbB receptor-specific rabbit polyclonal antibody was used at the same concentrations as above in combination with a mouse monoclonal antibody recognizing MAP2 (1:1,000 dilution), GFAP (1:1,000 dilution), pre-A4 (1:100 dilution), or CD45 (1:1,000 dilution). Signals were detected using Cy3- and fluorescein isothiocyanate-labeled reagents as previously described (29). The specificity of observed staining was confirmed by replacing the primary antibodies with nonimmune rabbit and mouse IgGs.

Confocal immunofluorescent images were obtained using a liquid-cooled CCD high-resolution monochromatic digital camera (model CH250; Photometrics, Tucson, AZ) coupled to a Leitz Orthoplan microscope. It was confirmed that this optical setup resulted in no fluorescent bleed-through between channels. Images were processed using IPLab Spectrum software (Scananalytics, Fairfax, VA).

**Immunoblotting**

Lysates for immunoblotting were prepared by homogenizing tissue in 19 volumes of ice-cold HES buffer (20 mM HEPES [pH 7.4]/1 mM EDTA/250 mM sucrose) containing 2 μg/ml aprotinin and 2 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using a modified Lowry method. Samples were resolved on 8% SDS-polyacrylamide gels and immunoblotted as previously described (29, 30). Immunoreactive species were identified by enhanced chemiluminescence.

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**RESULTS**

**ErbB4, but not erbB2 or erbB3, Immunoreactivity Is Specifically Associated with Neuritic Plaques in the Hippocampus of AD Patients**

The pattern of erbB2, erbB3, and erbB4 immunoreactivity was examined in the hippocampal formation of 11 patients with a pathologically confirmed diagnosis of AD and 5 cognitively normal age-matched controls (Table 1). The distribution of erbB staining in the hippocampus of the human controls was similar to that previously observed in rat hippocampus (29), confirming that the expression of all 3 erbB tyrosine kinases in humans was similar to that found in rat (see below).

In the dentate gyrus of both AD and control patients, erbB4 protein was detected as low-level diffuse staining of the granule cell layer (Fig. 1A). ErbB4 immunoreactivity was also present as punctate staining throughout the synapse-rich molecular layer (Fig. 1A), consistent with the reported association of this tyrosine kinase with postsynaptic densities (31, 32). In contrast to controls, all 11 AD patients also displayed numerous large erbB4-positive structures scattered throughout the dentate gyrus (Fig. 1A, arrowheads) and other regions of the hippocampal formation. These erbB4-positive structures colocalized with neuritic plaques (modified Bielschowsky stains; Fig. 1B), indicating that the plaques contain the erbB4 tyrosine kinase. ErbB4 immunoreactivity surrounding these plaque-like structures is associated with numerous irregularly shaped elements clustered around the amyloid composing the plaque core (Fig. 1C). Infrequently, a few erbB4-positive plaque-like structures were found in the

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**TABLE 1**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
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<th>Diagnosis</th>
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<td>M</td>
<td>AD</td>
<td>V/VI Frequent</td>
</tr>
<tr>
<td>2</td>
<td>78</td>
<td>F</td>
<td>AD</td>
<td>V/VI Frequent</td>
</tr>
<tr>
<td>3</td>
<td>77</td>
<td>M</td>
<td>AD</td>
<td>V/VI Frequent</td>
</tr>
<tr>
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<td>81</td>
<td>F</td>
<td>AD</td>
<td>V/VI Frequent</td>
</tr>
<tr>
<td>5</td>
<td>79</td>
<td>F</td>
<td>AD</td>
<td>V/VI Frequent</td>
</tr>
<tr>
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<td>72</td>
<td>M</td>
<td>AD</td>
<td>V/VI Frequent</td>
</tr>
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<td>88</td>
<td>F</td>
<td>AD</td>
<td>IV/VI Frequent</td>
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<tr>
<td>16</td>
<td>71</td>
<td>M</td>
<td>Control</td>
<td>I/VI Negative for plaques</td>
</tr>
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</table>

Abbreviations: AD, Alzheimer disease; M, male; F, female.
Fig. 1. Expression of erbB- and NRG-1-like immunoreactivity in the hippocampus of human patients with AD. A: ErbB4 immunoreactivity in an adjacent section of the hippocampus from this same patient shows that erbB4 staining is present in structures with a diameter similar to that of the neuritic plaques detected with modified Bielschowsky stains. B: Modified Bielschowsky stain of the hippocampus of an AD patient, demonstrating numerous mature neuritic plaques. C: Examination of erbB4-positive plaques at higher power shows that erbB4 immunoreactivity is associated with variably sized structures surrounding the plaque core (PC). D: ErbB4 immunoreactivity is also present in association with the rare neuritic plaques found in cognitively normal aged humans. E, F: Staining for erbB4 is abolished when the primary antibody is preincubated with the immunizing peptide (E), but not when preincubated with an unrelated peptide (F). G: ErbB2 immunoreactivity, like that for erbB3, is found on neuronal cell bodies and as punctate neuropil staining in the molecular layer; no erbB2 immunoreactivity is found in neuritic plaques. H: In the hippocampus of AD patients, erbB3 immunoreactivity is detected on neuron cell bodies (lower portion of the field) and as minimal punctate staining in the surrounding neuropil. ErbB3 staining is not seen in association with neuritic plaques. I: Higher power examination of neuritic plaques in sections stained for erbB2 shows little erbB2 immunoreactivity in these structures. PC, plaque core. J: Higher power examination of neuritic plaques in sections stained for erbB3 also show little evidence of erbB3 immunoreactivity in these structures. This preparation has been counterstained with Congo red to highlight the amyloid deposits within the plaque core. PC, plaque core. K: NRG-1-like immunoreactivity is evident in association with hippocampal pyramidal neurons, as punctate neuropil staining and in a neuritic plaque (arrowhead) in this section from an AD patient. L: Higher power view of an NRG-1-immunoreactive plaque shows that NRG-1 staining, like erbB4 staining, is associated with variably sized structures surrounding the plaque core (PC).
Neuregulin-Like Immunoreactivity Is Specifically Associated with Neuritic Plaques in the Hippocampus of AD Patients

To determine whether NRG-1 protein is also present within neuritic plaques, immunohistochemical staining of control and AD hippocampus was performed using an antibody specific for a common group of NRG-1 splice variants expressed in brain (transmembrane isoforms with an “a” variant carboxy terminus [33–35]; Carroll et al, unpublished observation). Since the antigen recognized by this antibody is not believed to be released from the cell of origin when the transmembrane precursor is proteolytically cleaved (34), this antibody marks cells synthesizing these NRG-1 isoforms. Neuronal staining was observed in the dentate gyrus and the CA3 region of the hippocampus in both control and AD cases (Fig. 1K).

This is in agreement with previous in situ hybridization demonstrations of NRG-1 mRNA expression in these cells (35, 36). The NRG-1 antibody also produced punctate labeling in adjacent synapse-rich regions, consistent with the pattern of NRG-1 immunoreactivity in equivalent regions in rat hippocampus (37). In AD brains there was additional NRG-1 labeling associated with neuritic plaques (Fig. 1K, arrowhead). These NRG-1-immunoreactive areas, like the erbB4-specific labeling, surround the plaque core (Fig. 1L).

To further verify the specificity of the NRG-1 and erbB4 antibodies used in this study and to assess whether the level of expression of either of these antigens is reproducibly altered in AD, lysates were prepared from the hippocampi of several of the control and AD cases that had been studied immunohistochemically. A protein of the expected 185-kD size was readily detected in immunoblots of lysates probed with the anti-erbB4 antibody (Fig. 2, left panel). No differences in the level of erbB4 expression were observed when signals obtained in control and AD hippocampal lysates were compared. Several NRG-1-like antigens, with masses of approximately 72 kD, 90 kD, and 110 kD, were also evident in lysates of control and AD hippocampi (Fig. 2, right panel). These NRG-1 species are slightly larger than the proteins detected in mouse cortex (see below), a distinction that may reflect either differences in the brain regions sampled in these 2 experiments or species-specific variability in the extensive post-translational modifications (primarily glycosylation) that occur in newly synthesized NRG-1 polypeptides (34). The level of expression of NRG-1 antigens in both control and AD hippocampi varied considerably, precluding any conclusion that total NRG-1 levels are altered in the hippocampus of AD patients.

ErbB4- and NRG-1-1-Like Immunoreactivity Is Associated in Part with Dendrites

To establish whether the punctate erbB4 and NRG-1 immunoreactivity in synapse-rich regions of the hippocampus was associated with dendrites, double label immunohistochemistry was performed on sections of hippocampus from AD patients using erbB4 or NRG-1...
antibodies in combination with an antibody recognizing the dendrite-specific protein MAP2. The distribution of these antigens was then compared using confocal microscopy. ErbB4 immunoreactivity was again evident as puncta (Fig. 3A), which were frequently observed to be arranged in rows within the neuropil. The MAP2 antibody labeled a meshwork of dendritic processes in this same region (Fig. 3B). A comparison of the distribution of erbB4 and MAP2 immunoreactivity demonstrated that the punctate erbB4 staining was frequently aligned along dendrites (Fig. 3C, D, arrows). NRG-1 immunoreactivity was similarly associated with MAP-2-positive dendritic processes (Fig. 3E–H). These findings demonstrate that erbB4 and NRG-1 immunoreactivity in human hippocampus is frequently distributed along the synapse-rich dendritic profiles present within the hippocampal molecular layer.

**ErbB4- and NRG-1-Like Immunoreactivity Is Differentially Associated with Cellular Elements within Neuritic Plaques**

To further characterize the cells in which erbB4 immunoreactivity within neuritic plaques is altered, double label immunohistochemistry was performed using the erbB4 antibody in combination with antibodies specific for the amyloid precursor protein (pre-A4, which marks a subpopulation of the dystrophic neurites in plaques), leukocyte common antigen (CD45), or the astrocytic intermediate filament protein (GFAP). Confocal digital microscopy was used to determine the relationship between erbB4 and each of these antigens. Pre-A4-positive dystrophic neurites were present throughout neuritic plaques, but there was little evidence of erbB4 expression within these processes (Fig. 4A–C). In contrast, a significant amount of the erbB4 immunoreactivity colocalized with CD45-positive microglia within plaques (Fig. 4D–F). Some of the remaining erbB4 labeling associated with neuritic plaques in the AD hippocampal formation was found in reactive astrocytes (Fig. 4G–I); however, this astrocytic expression of erbB4 was highly variable, with many plaques found to contain erbB4-negative astrocytes (data not shown). ErbB4 staining of astrocytes and microglia in the neuropil adjacent to and outside of neuritic plaques was much weaker than that observed for these same cell types within plaques, consistent with the observation that erbB4 immunoreactivity is enhanced in reactive astrocytes and microglia within zones of pathology in other disease states (22).

A comparison of immunoreactivity for NRG-1 and pre-A4 indicates that some of this growth factor is
ErbB4 immunoreactivity in neuritic plaques from human AD patients is primarily found in the reactive microglia and astrocytes surrounding the plaque core. A–C: Low power view of erbB4 (A) and amyloid precursor protein (B; pre-A4) labeling in a neuritic plaque. Merging of these staining patterns (C) shows little overlap, indicating that erbB4 immunoreactivity is largely distinct from pre-A4-positive dystrophic neurites. D–F: In this view of the periphery of a neuritic plaque, erbB4 staining (D) is seen to partially overlap with reactive macrophages (detected by labeling for CD45; E). Areas of overlap are evident as yellow staining (arrowheads) in the merged image presented in (F). G–I: ErbB4 immunoreactivity also is evident in reactive astrocytes within neuritic plaques. Note the overlap of some of the erbB4 staining (G) in this plaque periphery with staining for the astrocytic marker GFAP (H). Areas in which these 2 markers colocalize are evident as yellow staining (arrows) in (I). Scale bars: C, F, I = 40 μm.

potentially associated with dystrophic neurites within neuritic plaques (Fig. 5A–C, arrow). The majority of the NRG-1 expression found in these structures, however, colocalizes with CD45-positive microglia (Fig. 5D–F), with minimal NRG-1 staining evident in adjacent reactive astrocytes (Fig. 5G–I). We conclude that reactive microglia within neuritic plaques are a major source of NRG-1 protein within these structures.

ErbB4-, erbB2- and NRG-1-Like Immunoreactivity Is Associated with Neuritic Plaques in APP<sub>K670N;M671L</sub>/PS1<sub>M146L</sub> Double Mutant Mice

Transgenic mice coexpressing mutant APP<sub>K670N;M671L</sub> and PS1<sub>M146L</sub> genes develop numerous large neuritic plaques throughout the hippocampus (Fig. 6A), entorhinal cortex, and cerebral cortex (data not shown). As in the rat (29)
Fig. 5. Neuregulin-like immunoreactivity in human neuritic plaques is associated with reactive microglia and some dystrophic neurites. A–C: Staining for both NRG-1 (A) and amyloid precursor protein (pre-A4)-positive dystrophic neurites (B) is evident within neuritic plaques. Some structures within this plaque are labeled for both markers (C, arrowhead), demonstrating that at least some of these dystrophic neurites contain NRG-1. D–F: As seen in this view of a plaque labeled for both NRG-1 (D) and CD45 (E), the majority of the NRG-1 immunoreactivity in plaques is associated with the reactive macrophages arrayed around the plaque core (evident in the merged image in [F] as yellow staining; some of the positive cells indicated by arrowheads). G–I: Possible light NRG-1 staining (G) is associated with some of the reactive astrocytes (detected by their immunoreactivity for GFAP in [H]); these cells are indicated by arrowheads in the merged image presented in (I). Scale bars: C, F, I = 40 μm.

and human (see above), erbB4 immunoreactivity is present as diffuse staining of granule cells in the murine dentate gyrus and as punctate labeling in the adjacent molecular layer (Fig. 6B). In APP<sub>K670N,M671L</sub>/PS1<sub>M146L</sub> mutant mice, erbB4 labeling is associated with neuritic plaques in the hippocampal formation and in other areas of the telencephalon (Fig. 6B, arrows). Consistent with the erbB4 expression observed in human AD plaques, erbB4 specifically associates with structures located at the periphery of the dense plaque core in these double transgenic mice (Fig. 6C). In wild type littermates erbB4 is expressed in granule cells (Fig. 6D), pyramidal neurons of CA1, CA2, and CA3, and in the hilar neurons (data not shown); however, no plaques were observed by staining for erbB4 (Fig. 5D) or by modified Bielschowsky staining (data not shown) in control mice.

Granule cells in the dentate gyrus of both control and APP<sub>K670N,M671L</sub>/PS1<sub>M146L</sub> mutant mice express erbB3. ErbB4
Fig. 6. ErbB4, erbB2, and NRG-1 immunoreactivity is associated with neuritic plaques in the mid-hippocampal formation of 10-month-old transgenic mice expressing mutant APP (APP<sup>K670N,M781L</sup>) and PS1 (PS1<sup>M146L</sup>) genes. Three age-matched controls and 3 transgenic mice were examined for these studies. A: Modified Bielschowsky stains demonstrate the presence of multiple large plaque-like structures (arrows) in the brains of mice expressing these 2 transgenes. B: ErbB4-like immunoreactivity is associated with hippocampal pyramidal neurons and neuritic plaques (arrows) in a transgenic mouse brain. Magnification = ×100. C: A higher power view (×400) of erbB4 staining associated with neuritic plaques demonstrates that this immunoreactivity, like that associated with human plaques, is present in irregularly shaped structures surrounding the plaque core (PC). D: No plaques are
but not erbB3 immunoreactivity is associated with neuritic plaques (Fig. 6E, F). ErbB2 is diffusely expressed by cells in the granule layer, and in a punctate pattern in the molecular layer and colocalizes with neuritic plaques in this region (Fig. 6G). At higher magnification it is evident that erbB2 staining is closely associated with variably sized structures surrounding the plaque core (Fig. 6H). The expression pattern of NRG-1 in the dentate gyrus of transgenic mice closely resembles that of erbB4 and erbB2, with dense staining of the granule cell layer, punctate staining of the molecular layer, and dense immunoreactivity around plaque cores (Fig. 6I, J).

To assess whether the level of expression of either erbB4 or NRG-1 is altered in APP<sub>K670N</sub>;M671L/PSI<sub>M146L</sub> mice, lysates were prepared from the cerebral cortex and hippocampus of several transgenic animals and nontransgenic controls. A protein of the expected 185-kD size was readily detected in immunoblots of lysates probed with the anti-erbB4 antibody (Fig. 7, left panel), but there were no differences in the level of erbB4 expression in control versus transgenic mice. Several NRG-1-like antigens, with masses of approximately 56 kD, 71 kD, and 96 kD, were also identified in lysates of control and AD hippocampi (Fig. 7, right panel); these proteins are highly similar in size to the NRG-1 polypeptides present in rat spinal cord (52 kD, 75 kD, and 100 kD) (30). There were no significant differences in the levels of these NRG-1-like antigens in wild type compared to APP<sub>K670N</sub>;M671L/PSI<sub>M146L</sub> doubly transgenic mice.

**DISCUSSION**

NRG-1 proteins are believed to play a critical role in synaptogenesis and the survival of at least some neurons in the developing nervous system and may contribute to gliosis and inflammatory responses in the injured nervous system. These observations led us to hypothesize that NRG-1 and their erbB receptors continue to be expressed in the brain of cognitively normal aging humans and AD patients and that alterations in the expression of these molecules may occur in AD. In this study, we have shown that NRG-1, erbB2, erbB3, and erbB4 are indeed expressed in the hippocampus of aging patients and that the distribution of these molecules is altered in AD (Table 2), with erbB4 and NRG-1 immunoreactivity being associated with neuritic plaques.

In the hippocampus of both cognitively normal aged humans and AD patients, immunoreactivity for the NRG-1 receptor subunits erbB2, erbB3, and erbB4 was associated with numerous neuronal cell bodies in a pattern highly similar to that previously observed in optimally fixed tissue from adult rats (29). Staining for erbB2 and erbB4 was also evident as puncta throughout the synapse-rich molecular layer, suggesting that these 2 kinases are associated with the numerous dendrites of the hippocampal molecular layer (see below for further discussion of this point). In contrast to erbB2 and erbB4, erbB3 was associated with neuronal somas, but was largely absent from the molecular layer. These findings indicate that specific NRG-1 receptor subunits are localized to distinct compartments in at least some hippocampal neurons. This differential localization is significant as there are several important functional differences among these erbB kinases. First, NRG-1 does not bind directly to erbB2 (38). NRG-1 actions are instead mediated by binding to erbB3 or erbB4, with subsequent erbB2 tyrosine phosphorylation resulting when erbB2 heterodimerizes with either of these receptors (39); alternatively, erbB4 can homodimerize to form an active receptor (39). Further, erbB3 binds NRG-1 with an affinity an order of magnitude lower than that of erbB4 and, unlike erbB4, has little endogenous tyrosine kinase activity as 2 residues critical for autophosphorylation are “mutated” in this receptor (39). ErbB3 is also distinct in that it recruits some signaling molecules to the active signaling complex (e.g. phosphatidylinositol-3 kinase) that other erbB receptors cannot directly activate (39). Consequently, neuronal sensitivity to NRG-1 stimulation and the specific biologic response elicited may differ, depending on whether NRG-1 stimulation occurs at the cell body or at synapses.

Our proposal that the erbB2 and erbB4 immunoreactivity in the molecular layer represents synapse-associated accumulation of these kinases is consistent with the previously reported enrichment of these proteins in rat brain postsynaptic density fractions (31, 32). The NRG-1 antibody produced a similar pattern of staining in the hippocampal molecular layer, suggesting that this factor,
like its receptors, is also synapse-associated; NRG-1 at this location is likely presynaptic as NRG-1 protein is enriched in subcellular fractions containing synaptosomes and synaptic vesicles (37). Consistent with these suggestions, we have shown that some of the NRG-1 and erbB4 immunoreactivity in the hippocampal molecular layer is associated with dendrites. Previous analyses of synaptic density in the inner and outer molecular layers of the hippocampal dentate gyrus have demonstrated that these regions suffer a significant loss of synapses in AD subjects relative to the age-related losses that occur in normal controls (40, 41). We would emphasize, however, that at the level of examination used in this study, we cannot yet definitively establish whether synapse-associated erbB or NRG-1 immunoreactivity is diminished in the hippocampus of AD patients or APP_{K670N,M671L}/PS1_{M146L} mice.

These results indicate that erbB4 immunoreactivity is associated with cells surrounding the amyloid core of neuritic plaques. The widespread neuronal expression of erbB4 and its probable association with dendrites and its presence in neuritic plaques suggested that plaque-associated erbB4 represents accumulations of this kinase within neuronal processes disrupted by amyloid accumulation. However, we found little evidence that erbB4 colocalized with pre-A4-positive dystrophic neurites within plaques. This observation suggests the possibility that amyloid deposition results in a redistribution of erbB4 protein between damaged dendrites and the cell body; such a redistribution would be consistent with our finding that the accumulation of individual erbB receptors is restricted to specific neuronal compartments. Alternatively, amyloid-associated injury may involve portions of the dendritic tree proximal to the zones normally containing erbB4 protein. This latter possibility seems less likely, however, in light of the distribution of punctate erbB4 immunoreactivity throughout the neuropil immediately adjacent to the plaques examined in this study.

In contrast to dystrophic neurites, the reactive microglia and, to a lesser extent, the reactive astrocytes within neuritic plaques were readily labeled with the erbB4 antibody. ErbB4 immunoreactivity was minimal in microglia outside of the neuritic plaques. This increase in microglial erbB4 staining within neuritic plaques is reminiscent of the enhanced microglial erbB4 expression evident in active multiple sclerosis lesions (22) and within zones of CNS trauma (42), and suggests that microglial expression of erbB4 may be induced by multiple types of injury, possibly as part of the inflammatory response. ErbB4 staining was also not evident in non-plaque-associated astrocytes in the adjacent neuropil, consistent with our previous demonstration that astrocytes do not express detectable levels of this marker in normal rat hippocampus (29). We conclude that erbB4 expression is induced in the microglia and some of the astrocytes within neuritic plaques; this altered receptor expression likely results in alterations in microglial and astrocytic responses when these cells are stimulated by NRG-1.

In the developing peripheral nervous system, NRG-1 is synthesized by motor neurons and is believed to be transported along axons to the periphery, where it is released from the presynaptic terminus to act on the neuromuscular junction (14, 43). NRG-1 may be similarly transported along neuronal processes in the brain, leading to the expectation that NRG-1 immunoreactivity might accumulate in dystrophic neurites within neuritic plaques. We did observe occasional neurites in which NRG-1...
staining colocalized with pre-A4-positive dystrophic neurites. However, the majority of the damaged neurites within plaques did not label for NRG-1, indicating that these structures are likely a minor source of NRG-1. In contrast, NRG-1 immunoreactivity was quite evident in association with the reactive microglia in the plaques. These observations are consistent with previous reports that activated microglia (22, 44) are capable of expressing NRG-1. Multiple sources of NRG-1 are therefore potentially available to the microglia and astrocytes within neurotic plaques. As many of the cells in plaques express both erbB4 and NRG-1, it is likely that NRG-1/erbB signaling within these structures involves autocrine and/or paracrine stimulation. It is not yet clear how this autocrine/paracrine signaling would interact with the signaling mechanisms proposed to be activated secondary to γ-secretase mediated cleavage of erbB4 (25, 26).

As in human AD, NRG-1 and erbB4 immunoreactivity is present in association with neuritic plaques developing in mice coexpressing APPK670N/M711 and PS1M146L transgenes. However, staining for erbB2 is also evident in the plaques in APPK670N/M711/PS1M146L transgenic mice. It is currently unclear why erbB2 expression is present in the plaques of APPK670N/M711/PS1M146L transgenic mice and absent from these same structures in human AD. One possibility is that this distinction reflects the stage of plaque development in the transgenic mice we studied and that erbB2 expression may be lost from plaques in older animals with more advanced disease. Alternatively, the association of erbB2 immunoreactivity with neuritic plaques in APPK670N/M711/PS1M146L transgenic mice may reflect species-specific differences in the response to amyloid deposition and plaque development.

In conclusion, this study demonstrates for the first time that NRG-1, erbB2, erbB3, and erbB4 are expressed in the hippocampal formation of aged humans, and that the NRG-1 and erbB immunoreactivity in these hippocampi is associated with neuronal cell bodies and dendrites. The expression of both NRG-1 and erbB4 is also specifically associated with reactive cellular elements within neuritic plaques, suggesting that autocrine and/or paracrine interactions are important signaling mechanisms in these structures. It is not yet clear whether alterations in NRG-1 and erbB receptor distribution are essential events in the pathogenesis of AD or whether these changes occur as a response to the development of AD pathology. However, as the key alterations in NRG-1 and erbB expression observed in human AD are recapitulated in transgenic mice coexpressing mutant β-APP and presenilin-1 genes, it should be possible to experimentally manipulate signaling through the NRG-1/erbB signaling pathway in these animals and thus elucidate the role of these molecules in the pathogenesis of AD.

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