Regional Alterations in M₄ Muscarinic Receptor-G Protein Coupling in Alzheimer's Disease

CHRISTOPHER J. LADNER, GASTONE G. CELESLA, DEBRA J. MAGNUSON AND JOHN M. LEE

Abstract. Previous studies examining the functional status of cortical muscarinic cholinergic M₄ receptors have demonstrated an impairment in receptor-G protein coupling in Alzheimer's disease (AD) as measured by the inability of the receptor to form a high affinity agonist binding site. In order to investigate whether this alteration was a global phenomenon or a regional specific defect in signal transduction, we examined agonist binding at M₄ receptors in three brain areas (superior frontal cortex, Brodmann areas 8 and 9; primary visual cortex, Brodmann area 17; and the dorsal striatum) within the same brain in controls and moderate to severe AD cases. Competition binding studies using the M₄ antagonist [³H]pirenzepine (4 nM) in the presence of varying concentrations of the cholinergic agonist carbachol (50 nM to 1 mM) were performed in the presence and absence of GppNHp (100 μM), a non-hydrolyzable analog of GTP. In control membrane preparations, computer-assisted analysis of agonist-antagonist competition curves revealed that M₄ receptor agonist binding fit a two site model with high and low affinity states in all three brain areas in the absence of GppNHp but only a single site in the presence of GppNHp. This is consistent with the ternary complex model of G protein-linked receptors. In contrast, curves obtained from both cortical regions from AD brains fit a single site model with low affinity in the presence or absence of GppNHp. On the other hand, agonist binding data obtained from the dorsal striatum of AD cases exhibited a two site fit, similar to that seen in controls. The loss of M₄ high affinity agonist binding observed in AD is not a global defect, rather it appears to be restricted to discrete regions that are correlated with a relative abundance of neuritic/amyloid plaques but not diffuse plaques or neurofibrillary tangles.

Key Words: Alzheimer’s Disease; G protein; Muscarinic receptor.

INTRODUCTION

Neurons arising in the basal forebrain and septum provide the major source of cholinergic innervation of the human cerebral cortex and hippocampus (1). Lesioning studies in animals have demonstrated a critical role for these subcortical cholinergic projections in mediating processes of learning and memory (2). Furthermore, these cholinergic projections are decreased in individuals with Alzheimer's disease (AD) (3, 4). Although alterations in multiple neurotransmitter systems in AD (5, 6), a well-characterized, profound disruption of cholinergic neurotransmission is thought to partly underlie the mnemonic manifestations (7). Thus, initial attempts at therapeutic intervention have focused on enhancing the synaptic availability of acetylcholine (e.g. tacrine) and restoration of a cholinergic influence via exogenous cholinomimetics (e.g. muscarinic agonists). Unfortunately, most clinical trials have reported little to no improvement in memory (8–10). A critical assumption is made in this therapeutic strategy, namely that post-synaptic cholinergic receptor-mediated signal transduction remains intact in AD. Recent studies have called into question this crucial assumption.

Examinations of agonist binding to muscarinic receptors have demonstrated the existence of high and low affinity states (11) that are subject to modulation by GTP and GTP analogues, while muscarinic antagonist binding can be described by a single affinity value and is insensitive to GTP (12). In the presence of GTP, a reduction in high affinity agonist binding is observed (Fig. 1, left panel). Based on the ternary complex model of G protein-coupled receptors, this reduction in high affinity agonist binding represents a loss of receptor-G protein coupling. An additional consequence of receptor-G protein uncoupling is an attenuation of the ability of an uncoupled receptor to initiate signal transduction cascades.

Several studies examining the affinity states of the G protein-coupled M₄ muscarinic receptor have demonstrated a loss of high affinity agonist receptor sites in AD (13–16), suggesting that the M₄ receptor may be uncoupled from its G protein, G₄al, which is linked to phosphoinositide hydrolysis (17). This is further supported by the finding of decreased carbachol-stimulated M₄ receptor-mediated activation of phosphatidylinositol 4,5 bisphosphate (PIP₂) hydrolysis in the frontal cortex in AD compared to controls (16, 18). This occurred without a significant alteration in basal [³H]-PIP₂ turnover in AD. Furthermore, the decrease in agonist stimulation of [³H]-PIP₂ hydrolysis was correlated with a loss in high affinity agonist binding at the M₄ receptor (16) and occurred without a change in the level of G₄al α-subunit in AD (18). Thus, the loss of high affinity M₄ receptors is accompanied by a functional attenuation of downstream M₄-mediated signal transduction processes (Fig. 1, right panel).

Receptor agonist-antagonist binding competition studies allow for determining the ability of agonists to form
Fig. 1. A ternary complex of agonist, receptor, and G protein produces a conformational change in the receptor to increase its affinity for the agonist (high affinity site). The addition of guanine nucleotides (e.g. GTP or GppNHp) results in an exchange for GDP on the $\alpha$ subunit and an uncoupling of the G protein from the receptor. This is accompanied by a loss of high affinity agonist binding (left panel). In AD, the receptor is unable to couple to the G protein, manifested by a loss of high affinity agonist binding (right panel).

high affinity states of a receptor population, which represents the initial step in signal transduction. To date, studies utilizing this method to examine $M_1$ receptor-G protein coupling in AD have used brain tissue from the frontal, temporal, and parietal cortices, all of which exhibit to some degree the same characteristic pathological features of AD, namely neurofibrillary tangles (NFT) as well as neuritic core and diffuse amyloid plaques. Therefore, the present study examined $M_1$ receptor agonist affinity status in different brain areas in AD and aged controls in order to assess the relationship between the neuropathology and alterations in receptor-G protein interactions. The brain areas chosen included the superior frontal cortex (Brodmann areas 8 and 9), primary visual cortex (Brodmann area 17) and the dorsal striatum (caudate nucleus and putamen). These areas have been shown to contain $M_1$ receptors (19) as well as exhibit characteristic AD neuropathology (20–28) with the superior frontal cortex exhibiting extensive neuritic plaques and NFT, primary visual cortex with neuritic plaques but the relative absence of NFT, and the dorsal striatum with diffuse plaques and scattered NFT found only in large cholinergic interneurons. In addition, these areas differ in that the cholinergic projections to the cerebral cortex originate from neurons in the nucleus basalis of Meynert (1, 29–31) while cholinergic processes in the dorsal striatum arise from intrinsic interneurons within the nuclei (32–34). We hypothesized that if $M_1$ receptor uncoupling was related to pathological alterations associated with AD, it would be expected to exhibit region-specific alterations.

MATERIALS AND METHODS

Brain Tissue

Postmortem human brain tissue was obtained from the Loyola University Brain Bank. All samples were collected fresh at autopsy, flash frozen in isopentane at $-60^\circ$C, and stored at $-80^\circ$C. The diagnosis of AD is made based upon the National Institute of Aging consensus criteria which is based primarily on the density of senile plaques in the isocortex, as well as the presence of NFT and dystrophic neurons in the neocortex (35). The present study utilized samples from the superior frontal cortex (Brodmann areas 8 and 9), primary visual cortex (Brodmann area 17) and the dorsal striatum (caudate and putamen). The mean ages of controls and AD cases were 75.0 ± 3.2 years (SEM) and 83.1 ± 2.6, respectively, and did not differ significantly (p > 0.05). The mean postmortem intervals were 14.7 ± 1.5 hours for the control group and 14.5 ± 2.3 hours for the AD group (p > 0.05). All control cases were without clinical evidence of neurological impairment.

Binding Assay: Carbachol Inhibition of $^3$H-Pirenzepine Binding

Tissue specimens from four controls and seven AD cases were used in the study. Frozen brain tissue was thawed and homogenized on ice in 10 volumes (w/v) of 20 mM HEPES buffer (pH 7.5) and 1 mM EDTA. Initial homogenization was performed with a Tekmar electric homogenizer at setting 5 for
TABLE I
Carbachol Dissociation Constants (Kd) in the Presence and Absence of GppNHp

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Group</th>
<th>GppNHp</th>
<th>Kd-high affinity</th>
<th>Kd-low affinity</th>
<th>1 vs 2 site fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superior frontal</td>
<td>Control</td>
<td>–</td>
<td>5.8 μM</td>
<td>85 μM</td>
<td>2 site [F(22, 34) = 4.03; p &lt; 0.03]</td>
</tr>
<tr>
<td>cortex (n = 4)</td>
<td></td>
<td>+</td>
<td>n.d.</td>
<td>78 μM</td>
<td>1 site</td>
</tr>
<tr>
<td>(Brodman areas 8 and 9)</td>
<td>AD</td>
<td>–</td>
<td>n.d.</td>
<td>84 μM</td>
<td>1 site</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td>+</td>
<td>n.d.</td>
<td>71 μM</td>
<td>1 site</td>
</tr>
<tr>
<td>Primary visual</td>
<td>Control</td>
<td>–</td>
<td>1.1 μM</td>
<td>60 μM</td>
<td>2 site [F(25, 37) = 3.34; p &lt; 0.05]</td>
</tr>
<tr>
<td>cortex (n = 4)</td>
<td></td>
<td>+</td>
<td>n.d.</td>
<td>68 μM</td>
<td>1 site</td>
</tr>
<tr>
<td>(Brodman area 17)</td>
<td>AD</td>
<td>–</td>
<td>n.d.</td>
<td>61 μM</td>
<td>1 site</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td>+</td>
<td>n.d.</td>
<td>65 μM</td>
<td>1 site</td>
</tr>
<tr>
<td>Dorsal striatum</td>
<td>Control</td>
<td>–</td>
<td>1.1 μM</td>
<td>62 μM</td>
<td>2 site [F(26, 38) = 4.96; p &lt; 0.02]</td>
</tr>
<tr>
<td>(Caudate nucleus)</td>
<td>AD</td>
<td>–</td>
<td>2.6 μM</td>
<td>70 μM</td>
<td>2 site [F(26, 68) = 11.87; p &lt; 0.001]</td>
</tr>
<tr>
<td>and putamen (n = 4)</td>
<td></td>
<td>+</td>
<td>n.d.</td>
<td>66 μM</td>
<td>1 site</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td>+</td>
<td>n.d.</td>
<td>64 μM</td>
<td>1 site</td>
</tr>
</tbody>
</table>

Values were determined using SCAFIT component of LIGAND curve fitting program. n.d.: not detected.

two 15 second intervals. The homogenate was centrifuged at 49,000 g for 10 minutes at 4°C. Supernatant liquid was discarded, the pellet was resuspended with the above buffer and centrifuged as before. The pellet was washed and centrifuged twice in HEPES buffer prior to assay. Duplicate 1 ml suspensions of membranes, each consisting of 10 mg original wet weight of tissue in 20 mM HEPES buffer with 1 mM MgCl2 and without EDTA, were incubated for 1 hour in a shaker bath set at 25°C with 4 mM 1H-pirenzepine (New England Nuclear, Boston, MA) and 50 nM to 1 mM carbachol alone, or with 100 μM GppNHp (Sigma, St. Louis, MO). Samples were then rapidly filtered on to Whatman GF/B paper and rinsed with 2 ml cold HEPES buffer using a Brandel Cell Harvester. Filters from each sample were then placed in a plastic scintillation vial with 6 ml of Scintiverse liquid scintillation fluid (Fisher Scientific, Pittsburgh, PA). Scintillation counting was conducted 18 hours later using a Beckman LS6000LL scintillation counter.

Data Analysis

The specific binding was determined by subtracting the amount of 1H-pirenzepine bound in the presence of 1 mM carbachol from total binding in each sample. The resulting curves from controls and AD patients were tested to determine whether the data fit to a one or two site model using a nonlinear iterative curve fitting computer program LIGAND (36). This program determines whether a particular model (e.g. one site versus two site) is significantly better fit to the data by comparing the sum of squares using a partial F-test. Binding data were analyzed individually for each case and simultaneously fit as a group for control and AD cases using the SCAFIT component of the LIGAND program. The Kd-high, Kd-low, and receptor concentrations were also calculated by this program. Amyloid plaque and NFT counts were measured using the modified Bielschowsky method and were expressed as the mean ± SEM per high powered field (HPF, 200X). Statistical analyses of plaque and NFT densities were performed using a two-tailed Student’s t-test. In addition, the relationship between the neuropathological findings and M1 receptor-G protein coupling was examined using a Fisher’s exact probability test. This test was employed because of the nominal nature of the agonist binding variable. That is, M1 receptors exhibited either two site binding (coupled) or a single site (uncoupled). The analysis utilized the binding and histological data derived from brain samples from all three brain regions in controls and AD cases. Comparisons were made between the proportion of individual control and AD brain samples exhibiting one versus two site fit with the presence or absence of neuritic plaques, diffuse plaques (>10/HPF) or NFT.

RESULTS

The composite curves and Kd values for carbachol binding in control and AD brains are presented in Table 1 and Figure 2. The control membrane curves best fit a two affinity state model for carbachol binding in the superior frontal cortex [F(22, 34) = 4.03; p < 0.03], primary visual cortex [F(25, 37) = 3.34; p < 0.05], and dorsal striatum [F(26, 38) = 4.96; p < 0.02] at the M1 receptor. The addition of GppNHp (100 μM) to control membranes produced both a rightward shift and increase in the slope of the competition curve, which resulted in a best fit to a single, low affinity binding site (Fig. 2A).
Again, this is consistent with a ternary complex model of G protein-linked receptors. Therefore, M1 receptors retain their ability to couple to G proteins in control post-mortem tissue under the conditions described. Under these conditions, there was a mixed population of high and low affinity states of the M1 receptor, with approximately 20–30% of M1 receptors in the high affinity state for all three control brain areas examined (data not shown).

On the other hand, competition curves obtained from AD superior frontal cortex and primary visual cortex failed to fit a two site model. Curve fitting revealed a single binding site which corresponded to the low affinity site observed in control membranes (Table 1). The addition of GppNHp had no further effect on M1 receptor agonist binding characteristics in AD superior frontal (Fig. 2B) and primary visual cortices. However, carbamol binding to the M1 receptor in the dorsal striatum of AD patients exhibited a highly significant best fit with a two site binding model [F(26, 68) = 11.87; p < 0.001], similar to controls. Thus, in AD there appears to be a regional alteration in M1 receptor-G protein coupling, with superior frontal and primary visual cortices being affected with the dorsal striatum being spared. No significant difference in the number of M1 receptors was observed between AD and control brains in any region, although the dorsal striatum contained a relatively higher number of receptors (Bmax) relative to cortical areas (data not shown).

The results of microscopic examination of sections from the regions used in the binding study for AD cases only are presented in Figure 3. In AD sections examined, total senile plaques had the following distribution pattern: superior frontal cortex (23.7 ± 7.9; mean per HPF ± SEM) > primary visual cortex (10.7 ± 1.9) > dorsal striatum (5.8 ± 0.8). Neurofibrillary tangles were most abundant in superior frontal cortex (2.4 ± 1) and extremely rare in the primary visual cortex (0.3 ± 0.2) and dorsal striatum (0.3 ± 0.2). The number of neuritic/core plaques, however, were found in similar densities in both superior frontal cortex (5.2 ± 1.5) and calcarine cortex (5.0 ± 1.9), while absent from the dorsal striatum. Examination of control brain sections demonstrated an absence of neuritic plaques and NFT with low levels of diffuse plaques in superior frontal cortex (0.3 ± 0.3), primary visual cortex (1.7 ± 1.0) and dorsal striatum (0.5 ± 0.5).

These findings suggest that the loss of high affinity agonist binding at the M1 receptor is associated with the presence of neuritic plaques. In order to further analyze this relationship, a Fisher's exact probability test was utilized to compare the proportion of brain samples exhibiting one site fit (uncoupling) with the incidence of neuropathology. This analysis confirmed that the observed proportion of brain samples exhibiting both a loss of high affinity agonist binding at the M1 receptor (one site fit) and the presence of neuritic plaques was significantly greater (p = 0.04) than would be expected if the two variables were not correlated. Similar analysis of the number of diffuse plaques or the presence of NFT failed to demonstrate a significant association between loss of high affinity agonist binding at the M1 receptor (see Table 2).

Fig. 3. A. Total plaques. Superior frontal cortex and primary visual cortex had significantly greater total numbers of plaques than the dorsal striatum (p < 0.05). B. Neuritic/core plaques. Superior frontal cortex and primary visual cortex both had significantly greater numbers of neuritic/core plaques than the dorsal striatum (p < 0.01). C. NFT. Superior frontal cortex exhibited significantly greater numbers of NFT than either primary visual cortex or dorsal striatum (p < 0.05). Data is expressed as the mean ± SEM per HPF (200X) using the modified Bielschowsky silver stain.
| TABLE 2 |
| Coincidence of M, Receptor Uncoupling and Neuropathological Changes |

- A. One site fit (uncoupled receptor) vs neuritic plaques, p = 0.04*  
- B. One site fit vs diffuse plaques, p = 0.34  
- C. One site fit vs NFT, p = 0.25

*p values calculated using Fisher's exact probability test, total n = 33 (11 subjects × 3 brain regions).  
* p < 0.05

**DISCUSSION**

The findings of the present study are consistent with previous studies in that there was a significant loss of high affinity agonist binding at the M1 receptor in neocortical areas exhibiting typical AD pathological changes (18-21). The loss of high affinity agonist binding at the M1 receptor in AD suggests that the receptor is uncoupled from its G protein and that cholinergic signal transduction is impaired. Although previous studies examining uncoupling in AD demonstrated a loss of high affinity agonist binding at the M1 receptor, these earlier reports indicated that carbachol competition for 3H-pirenzepine binding still exhibited a two site best fit. In the present study, the uncoupling in AD was so severe in cortical areas that the best fit for carbachol binding was shifted from two sites to a single low affinity site. These results indicate that post-synaptic cholinergic transduction mechanisms are profoundly impaired in certain cortical areas in AD and may in part explain the failure, thus far, of cholinomimetic pharmacotherapy.

In addition to the cholinergic system, receptor-G protein uncoupling has been reported in other neurotransmitter systems in AD. Uncoupling has been reported at D5 dopamine receptors in human frontal cortex from AD cases (37) and at β adrenergic receptors in cultured skin fibroblasts from AD patients (38). These receptors are coupled to Go13, which stimulate adenyl cyclase. It has been demonstrated that a significant reduction in G protein activation of adenyl cyclase occurs in the frontal cortex in AD (39). On the other hand, serotoninergic 5HT1A and adrenergic α2 receptors in the frontal cortex remain coupled to their G proteins (40, 41). Studies examining the levels of G protein a subunits have failed to detect AD-associated changes (18, 42). Thus, receptor-G protein uncoupling does not appear to be related to a particular neurotransmitter or signal transduction cascade since coupling to both adenyl cyclase (via Go) and phosphoinositol hydrolysis (via Gq) systems appear to be affected in AD.

The alteration in M1 receptor-G protein coupling observed in AD is not a global defect, rather it appears to be localized to regions of the brain with significant neuropathological findings. M1 receptor-G protein uncoupling was observed in the superior frontal cortex and primary visual cortex. While both of these areas exhibit neuritic/core plaques in AD, NFT are rare in the primary visual cortex (20, 21). In the present study, M1 receptor-G protein coupling remained intact in the dorsal striatum, an area characterized by diffuse amyloid plaques, scattered NFT, and an absence of neuritic plaques (27, 28). Thus, the alterations observed in M1 receptor-G protein coupling in AD in the present study appear to be best correlated with neuritic/core plaques and not diffuse plaques or NFT formation. Furthermore, preliminary data from muscarinic agonist binding in four mild AD cases suggests that the alteration in M1 receptor-G protein coupling is present in earlier stages of the disease and does not represent an end stage phenomenon (personal observation).

The finding of an association of M1 receptor-G protein uncoupling with the presence of neuritic plaques is in agreement with what is known regarding the receptor-coupled processing of beta amyloid precursor protein (β-APP). Carbachol stimulation of M1 and M6 cholinergic muscarinic receptors promotes the release of a soluble, non-amyloidogenic fragment of β-APP (43, 44). These findings implicate cholinergic neurotransmission in the processing of β-APP to promote formation of the non-amyloidogenic APP, fragments. Conversely, a loss of M1 receptor-G protein coupling may be a pathological factor in the formation of neuritic plaques in AD since attenuation of the APP-directed processing may increase the substrate availability for the amyloidogenic pathway.

In the present study, M1 receptor uncoupling was restricted to the cortical areas examined. Examination of autopsy specimens obtained from AD brains with an antibody directed against muscarinic receptors demonstrated an increase in the number of reactive astrocytes expressing muscarinic receptors in cortical grey matter (45). Interestingly, these astrocytes were often associated with neuritic plaques. Astrocytes produce β-amyloid as well as regulate glutamate neurotransmission, two substances which have been implicated in the pathogenesis of AD. In fact, since M1 receptor activation has been shown to increase the amount of non-amyloidogenic fragments of β-APP, then the uncoupling of these receptors on astrocytes may result in increased β-amyloid production.

Although it has been presumed that the loss of subcortical cholinergic projections plays a central role in the dementia observed in AD, the failure of cholinomimetic pharmacotherapy, alterations in other neurotransmitter systems and the observation of receptor-G protein uncoupling at the predominant cortical muscarinic receptor subtype suggests that dementia may result from an interaction of several different processes. In light of the data implicating M1 receptor-mediated signal transduction in the processing of β-APP, the loss of subcortical cholinergic projections to the cortex in conjunction with im-
paiments of M1-mediated signal transduction associated with AD may exert a synergistic influence on the development of neuritic plaques. Furthermore, the present study suggests that cholinomimetic pharmacotherapy which is unable to overcome the receptor-G protein uncoupling will be ineffective for the treatment of AD. On the other hand, selective activators of phospholipase C, which bypasses the muscarinic receptor, may possibly have some utility for both the prevention of amyloid plaque deposition as well as the amelioration of the clinical symptoms of dementia.

REFERENCES


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